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Ah Receptor: Relevance of Mechanistic Studies to Human Risk Assessment

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Studies of the toxic actions of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in numerous animal models and in human and animal cells in culture have established that the most characteristic pathologic lesions produced by this compound result from events initiated by the interaction of TCDD with a specific intracellular receptor protein, the *Ah* receptor. Although most research on the interaction of TCDD with the *Ah* receptor has focused on establishing involvement of this receptor complex in specific toxic responses, recent application of modern cell and molecular biology techniques is yielding new insights into the mechanism(s) of signal transduction. Elucidation of these mechanisms is essential for understanding the molecular basis of the cell and species specificity which is a hallmark of TCDD toxicity. This knowledge should provide the framework for development of a more toxicologically relevant risk assessment model.

Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the extensively studied prototype for the halogenated aromatic compounds (HACs), a large group of environmental toxicants that includes the dibenzo-*p*-dioxins, dibenzofurans, biphenyls, and azo- and azoxybenzenes (*1–3*). Studies in various animal models and in human and animal cells in culture indicate that TCDD and isosteric HACs produce a characteristic pattern of pathologic lesions separable into two major categories: those that are widely expressed such as thymic atrophy, teratogenesis, and wasting; and species-specific responses including hepatotoxicity, edema of the pericardium, and hyperkeratosis and chloracne (*4*). Hyperkeratosis and chloracne are responses commonly observed in humans (*5,6*). Other reported clinical abnormalities include weight loss, impaired liver function, hepatic porphyria, general malaise, and peripheral neuropathies (*7*). Suppression of specific T-lymphocyte functions has been reported in individuals who ingested PCB-contaminated rice oil (*8–10*). Chlorinated dibenzofurans present in the PCB mixture are the suspected immunosuppressive agents (*8–11*).

Many of the actions of TCDD and related HACs on target cells are mediated by a specific intracellular binding protein (to be designated in this report as the *Ah* receptor) (*2,5*). Studies carried out in inbred murine strains have established that the *Ah* receptor mediates the TCDD-dependent induction of a battery of genes coding for cytochrome P_1-450 and other enzymes primarily involved in xenobiotic metabolism (*2*). In epithelial cells from certain target organs the *Ah* receptor is postulated to regulate a second gene battery encoding proteins involved in the control of cell proliferation and differentiation (*2,12,13*). Genetic analysis of TCDD-induced epidermal hyperplasia in haired and hairless strains of inbred mice has demonstrated that this *Ah* receptor-dependent response occurs only in the presence of a second regulatory gene (designated *hr*) (*14*).

Skin and Thymus Toxicity: *In Vitro* Models

Several *in vitro* model systems have been developed and used for study of the mechanisms of TCDD toxicity to skin (*13,15–18*) and thymus (*19–21*). Analysis of the concentration-dependence and stereospecificity requirements for toxic responses elicited by TCDD and other HACs in the various *in vitro* models has confirmed the involvement of the *Ah* receptor; however, specific transductional events triggered by the reversible binding of HAC agonists with the *Ah* receptor remain to be elucidated. Most studies have been largely correlative and have not investigated the molecular determinants of toxicity. Such information is essential for providing a mechanistic basis potentially useful for risk analysis. This is perhaps best demonstrated by the studies cited above in hairless mice in which it was shown that at least two regulatory genes (*Ah*, the putative structural gene locus for the *Ah* receptor, and *hr*) are required for the expression of toxicity; i.e., the presence of the *Ah*

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receptor in skin target cells is obligatory, but not sufficient to elicit a toxic response in murine skin (*2,14*).

Skin

Cultures of normal human epidermal cells and human squamous cell carcinoma (SCC) lines have been established in this laboratory as models for TCDD-induced epidermal hyperkeratinization (*22*). Treatment of newly confluent cultures of epidermal cells with TCDD results in an increase in the relative proportion of more highly differentiated cells as judged by histologic examination, decreases in the number of basal proliferating cells, and increases in the number of both envelope competent cells and cells with highly cross-linked cornified envelopes (*23*). This response observed *in vitro* appears to be analogous to the epidermal hyperkeratinization reported in both animal models (*14*) and in humans exposed to TCDD-contaminated 2,3,5-trichlorophenol (*24*).

Potential biochemical mechanisms for this TCDD-induced hyperkeratinization response have been studied in human SCC lines that display predominantly either enhanced differentiation (line SCC-12F) or altered proliferation (line SCC-9) in the presence of TCDD (*13*). Evidence obtained from these models indicates that TCDD-induced changes in growth and differentiation result from a complex interaction of the *Ah* receptor, with at least three other receptor systems involved in normal physiologic regulation of epithelial cell proliferation (*13*).

EGF Receptor

Interaction of the Ah receptor with one of these receptor systems, the EGF receptor, has been studied in some detail (*25,26*). In normal human epidermal cells it was observed that TCDD produced a concentration-dependent decrease in high-affinity EGF binding (*23*). This response was only elicited by *Ah* receptor ligands and preceded detectable TCDD-dependent modulation of terminal differentiation. Analysis carried out with SCC-12F cells indicated that TCDD through interaction with its cognate receptor (the *Ah* receptor) produced a sustained decrease in high-affinity EGF receptor binding but had no effect on the low-affinity EGF receptor binding (*25*). A number of lines of evidence suggested that modulation of EGF receptor binding was one of the determinants of TCDD-dependent epidermal hyperkeratinization observed *in vitro* (*25,26*).

Based largely on the similarity between the values for the calculated half-life (20 hr) for the reduction in high-affinity EGF binding in TCDD-treated SCC-12F cells (*25*) with the reported half-life (16 hr) for EGF receptor turnover in the human epidermoid carcinoma line A431 (*27*), it was postulated that TCDD altered the turnover of the EGF receptor in SCC-12F cells, possibly through suppression of the EGF receptor structural gene (*13*). Recent studies in this laboratory (*28*), however, indicate that TCDD treatment does not alter EGF receptor gene transcription or processing of the EGF receptor RNA transcript (Fig. 1). Potential posttranscriptional actions of TCDD on EGF receptor turnover have not been examined.

Actions on Thymic Epithelial Cells

Studies carried out in congenic and specific strains of inbred mice indicate that toxic responses to TCDD in several immune system target cells is mediated by the *Ah* receptor (*29–32*). Genetic analysis of TCDD-induced thymus atrophy indicates that a second regulatory locus is not required for the production of this *Ah* receptor-dependent response (e.g., thymic atrophy occurs in both haired and hairless C57BL/6 mice) (*2*). Several lines of evidence led to the proposal that thymic epithelium was the target cell for both TCDD-induced thymus atrophy (*29,30*) and enhanced T-suppressor cell activity (*2,31,33*). Previous studies carried out in this laboratory (*20*) demonstrated that TCDD can act directly on murine thymic epithelial monolayers to suppress the maturation of cocultured thymocytes. It was postulated that impaired thymocyte maturation could lead to increased thymocyte death and the depletion of cortical thymocytes characteristic of TCDD-induced thymic atrophy (*12*).

In a subsequent investigation, the actions of TCDD on cultures of epithelial cells established from human thymus specimens were characterized (*21*). The parameters examined included the induction of the cytochrome P_1-450 monooxygenase activities, 7-ethoxycoumarin *O*-deethylase (ECOD) and 7-ethoxyresorufin *O*-deethylase (EROD), and modulation of HuTE-dependent thymocyte responsiveness to the mitogens concanavalin A (Con A) and phytohemagglutinin (PHA). TCDD was found to induce the measured cytochrome P_1-450 activities and to suppress HuTE-dependent thymocyte maturation. The concentration-dependence and stereospecificity (as judged by the relative activities of chlorinated dibenzo-*p*-dioxin and dibenzofuran isomers) for both responses elicited by TCDD in HuTE cells indicated involvement of the *Ah* receptor (*21*). Comparison of TCDD-dependent induction of cytochrome P_1-450 and impaired thymocyte maturation in several strains of HuTE cells indicated significant interstrain differences in maximally inducible ECOD and EROD activities (Fig. 2 and Table 1) which did not appear to directly correlate with the measured concentration of the *Ah* receptor in HuTE cytosol. In certain HuTE strains treated with TCDD, differences in both sensitivity and magnitude were observed for the impaired HuTE-dependent thymocyte maturation (Fig. 3). Significant differences in sensitivity for the induction of ECOD activity were not seen (Fig. 2).

These observations on the actions of TCDD in HuTE cells suggested that human thymus is a potential target for TCDD and isosteric HACs. The validity of this *in vitro* model system as a predictor of toxicity is supported by recent reports of altered cell-mediated immunity in individuals exposed to chlorinated dibenzofurans (*11,12*). In addition, the *in vivo* dose of TCDD that

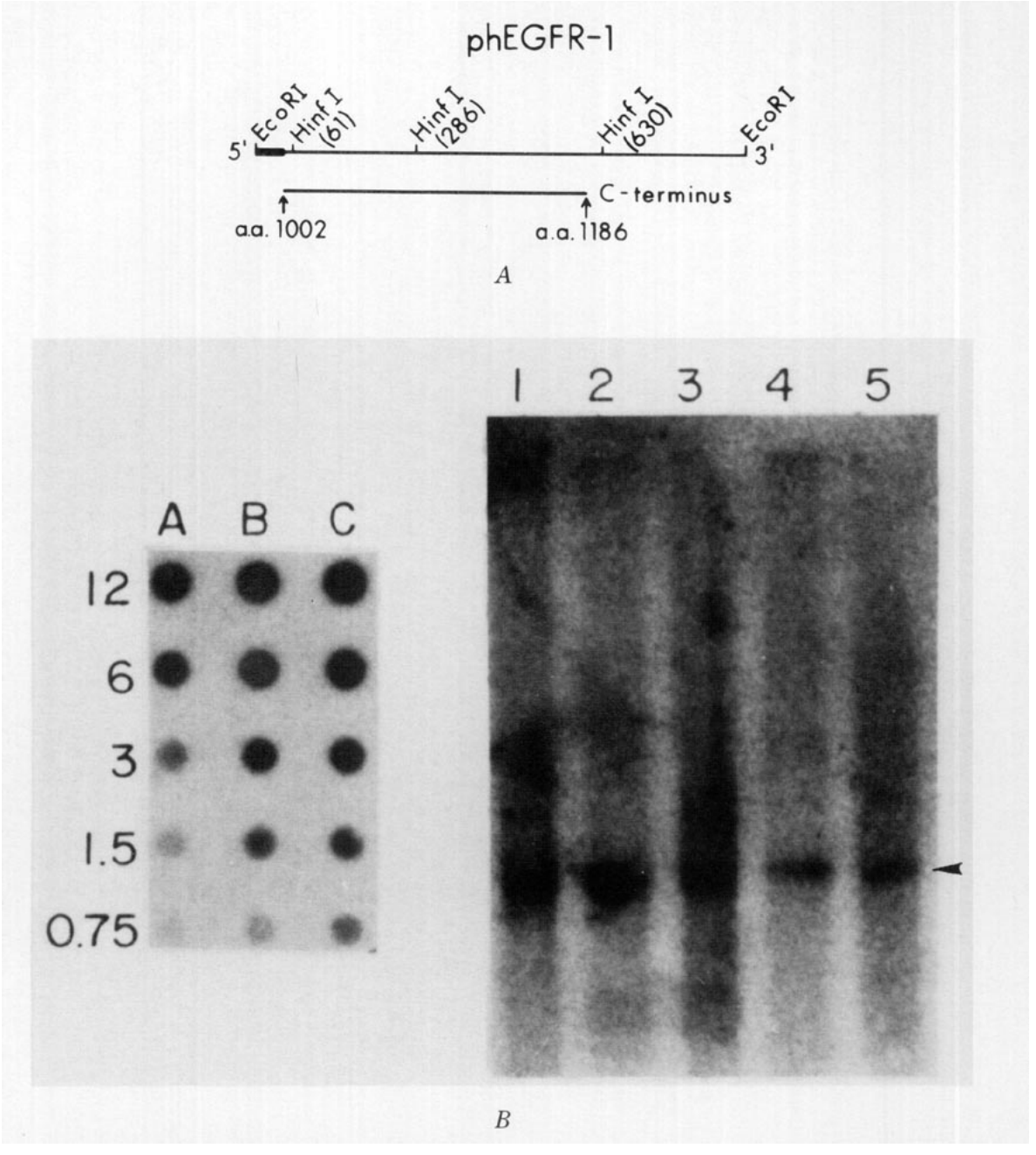

FIGURE 1. Filter hybridization analysis of total RNA from control and TCDD-treated SCC-12F cells. (*A*) Restriction sites of 889 bp cDNA probe encoding the 3' end of the human EGF receptor gene [designated clone phEGFR-1 (*41*)]. (*B*) Left: total RNA prepared from control and TCDD-treated (10 nM, 48 hr) cells was applied to nitrocellulose filters, hybridized to nick-translated phEGFR-1, and analyzed by autoradiography. Lane A, no treatment; Lane B, 0.01% DMSO (control); Lane C, 10 nM TCDD. The numbers in the first column indicate the amount of RNA (μg) applied to each well in the corresponding row. Right: northern analysis of total RNA. Lane 1, no treatment; Lane 2, 0.01% DMSO (48 hr); Lane 3, 10 nM TCDD (48 hr); Lane 4, 0.01% DMSO (72 hr); Lane 5, 10 nM TCDD (72 hr). The arrow indicates EGF receptor mRNA (6 kb). Taken in part from Osborne et al. (*28*).

promises host resistance and produces thymic atrophy in C57BL/6 mice is similar to concentrations shown to be active in both cultured murine thymic epithelial (*20*) and HuTE (*21*) cells. At least three conclusions relevant to the assessment of the toxic potential of HACs can be drawn from the studies carried out in the HuTE cell culture model system: (a) the *Ah* receptor mediates the biochemical and toxic actions of TCDD on an epithelial target cell population derived from human thymus; (b) HuTE strains established from thymus samples from difference individuals can differ markedly in sensitivity to the immunotoxic actions of TCDD; and (c) quantitation of the *Ah* receptor content (or measurement of cytochrome P_1-450 inducibility, a widely accepted *Ah* receptor-mediated

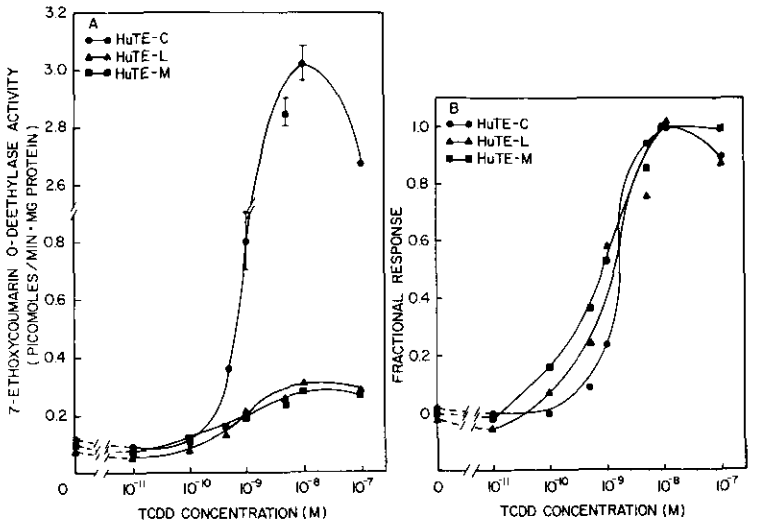

FIGURE 2. (*A*) Log dose-response curves for the induction of ECOD activity by TCDD in three strains of HuTE cells. Each point represents the mean ± SEM of three experiments. Points without error bars have an SE less than the symbol size. (*B*) Fractional dose-response curves of the data in (*A*). The mean control activity was equated to zero, the mean maximally induced activity to 1.0, and the intermediate responses were calculated as a fraction of the maximal response. The EC_{50} values for the three strains are 2.0 nM (HuTE-C), 0.9 nM (HuTE-L), and 0.8 nM (HuTE-M). From Cook et al. (*21*).

response) is not necessarily an accurate quantitative predictor of toxic susceptibility.

Conclusions

A central focus of most studies on the mechanisms of toxicity of HACs in various animal and cell culture model systems has been at the level of the *Ah* receptor. Receptor mediated actions occur through a specific sequence of events: reversible binding of the ligand (effector) to its cognate receptor (recognition); transmission of the binding signal into a molecular effector system (transduction); and generation of a response (*34*). Much is known implicating the *Ah* receptor in various

Table 1. *Ah* Receptor concentrations and cytochrome P_1-450 monooxygenase activities in strains of human thymic epithelial cells in culture.

Human thymic epithelium strain (donor's age, sex)	*Ah* receptor, fmole/mg protein[c]	7-Ethoxycoumarin *O*-deethylase, pmole/min·mg protein[a]			7-Ethoxyresorufin *O*-deethylase, pmole/min·mg protein[a]		
		Treatment[b]			Treatment[b]		
		DMSO	DCDD	TCDD	DMSO	DCDD	TCDD
C (16 years, female)	16.2 ± 1.6	0.160 ± 0.005	0.294 ± 0.020	4.902 ± 0.109*†	0.111 ± 0.006	0.312 ± 0.020*	1.585 ± 0.020*†
D (9 months, male)	28.3 ± 3.3	0.120 ± 0.025	0.263 ± 0.004*	0.443 ± 0.035*†	0.146 ± 0.033	0.443 ± 0.031[d]	0.840 ± 0.014*†
H (2 months, male)	15.2 ± 1.1	0.139 ± 0.009	0.233 ± 0.014	656 ± 0.083*†	0.066 ± 0.006	0.093 ± 0.010	0.138 ± 0.009*†
K (3.5 years, male)	22.4 ± 2.5	0.179 ± 0.003	0.237 ± 0.029	0.593 ± 0.114*†	0.025 ± 0.006	0.024 ± 0.004	0.067 ± 0.017
L (2 years, male)	48.8 ± 0.4	0.091 ± 0.021	0.093 ± 0.021	0.292 ± 0.046*†	0.045 ± 0.007	0.068 ± 0.002	0.121 ± 0.001*
M (5 years, male)	18.6 ± 0.3	0.084 ± 0.001	0.150 ± 0.003*	0.231 ± 0.009*†	0.104 ± 0.010	0.119 ± 0.006	0.129 ± 0.003
N (4 years, female)	10.9 ± 0.1	0.060 ± 0.018	0.188 ± 0.072	24.926 ± 0.477*†	0.227 ± 0.002	0.225 ± 0.022	4.701 ± 0.088*†
O (13 years, female)	6.5 ± 0.5	0.085 ± 0.017	0.165 ± 0.006	1.072 ± 0.061*†	0.100 ± 0.018	0.105 ± 0.003	0.271 ± 0.005*†
P (6 years, male)	8.7 ± 0.1	0.131 ± 0.008	0.552 ± 0.036	6.497 ± 0.482*†	0.065 ± 0.010	0.115 ± 0.007	0.778 ± 0.045*†

Abbreviations used: DMSO, dimethyl sulfoxide; DCDD, 2,7-dichlorodibenzo-*p*-dioxin; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.
[a] Values are expressed as the mean ± SE of three experiments.
[b] Human thymic epithelium was treated for 3 days with either 0.1% DMSO, 1×10^{-6}M DCDD, or 1×10^{-8}M TCDD.
[c] Values are expressed as the mean ± SE of two or three determinations. Cytosol was incubated with ligand for 2 hr at 20°C.
* Significantly different from control value ($p < 0.01$).
† Significantly different from DCDD ($p < 0.01$).

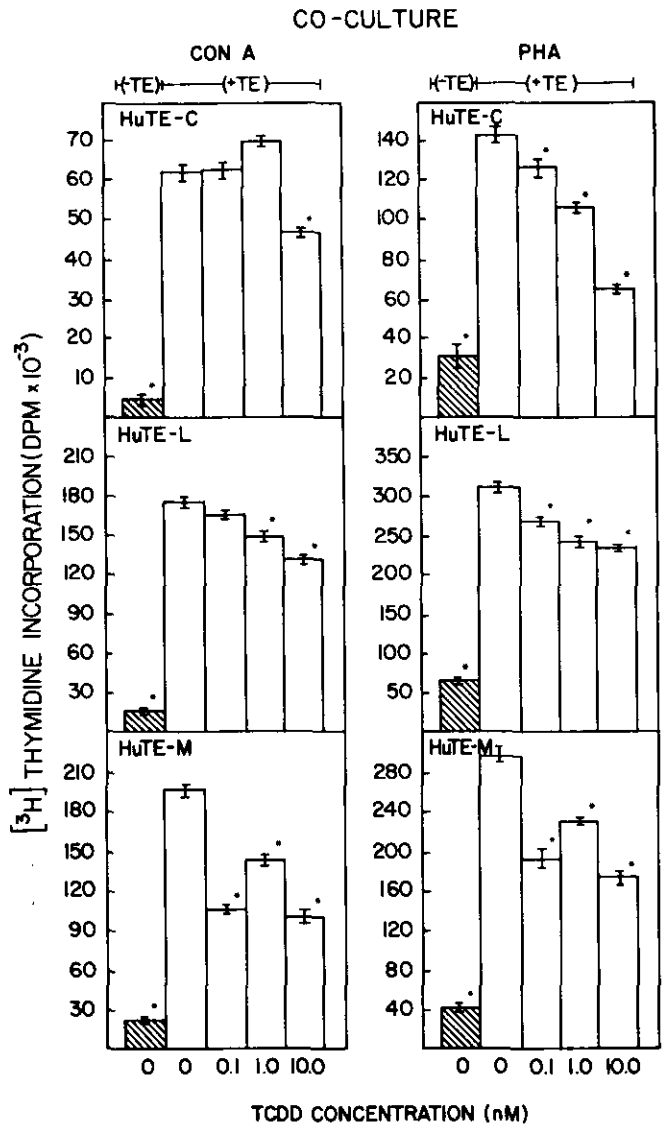

FIGURE 3. Con A- and PHA-dependent mitogenic responses of human thymocytes cocultured on HuTE monolayers. Each bar represents the mean ± SEM of four determinations. Asterisks indicate significant difference ($p < 0.01$) from the control cells (thymocytes cocultured on DMSO-treated TE monolayers). The hatched bars represent thymocytes not cocultured on HuTE monolayers. From Cook et al. (*21*).

biochemical and toxic responses; however, until recently, little was known about specific transductional mechanisms. Studies by Jones et al. (*35–37*) have resulted in significant advances in understanding the molecular mechanisms of the TCDD-dependent induction of cytochrome P_1-450 in murine hepatoma lines. These investigators have identified, cloned, and mapped the upstream regulatory elements for the cytochrome P_1-450 structural gene. These elements consist of a promoter (the binding site for the RNA polymerase complex), an inhibitory domain that blocks promoter function, and at least two dioxin-responsive elements, the putative nuclear acceptor sites for TCDD-*Ah* receptor

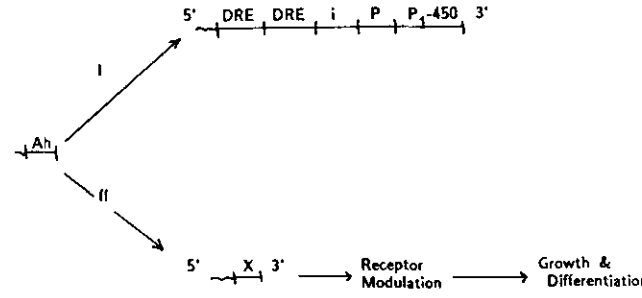

FIGURE 4. Proposed model for the regulation of multiple responses by the *Ah* receptor. DRE, dioxin-responsive regulatory elements (putative TCDD-*Ah* receptor DNA binding sites); i, inhibitory domain; P, RNA polymerase binding site; X, growth regulatory gene controlled by the TCDD-*Ah* receptor complex. Taken in part from Jones et al. (*36*).

complexes (*35*) (Fig. 4). The dioxin-responsive elements are located more than 1500 base pairs upstream from the cytochrome P_1-450 promoter (*35*), have the properties of transcriptional enhancers (*36*), and maintain responsiveness to TCDD when transfected into either heterologous murine tissue or a human mammary epithelial cell line (*36,37*).

Qualitatively similar observations have been reported for the regulatory region for the cytochrome P_1-450 gene isolated from C57BL/6 mouse liver (*38*). Dioxin responsive elements regulating the TCDD-dependent expression of the rat cytochrome P-450c gene also have been identified (*39*). Jaiswal et al. (*40*) compared the nucleotide sequences of the human and murine cytochrome P_1-450 genes and found four highly homologous boxes between these genes in the TATA box promoter region. These observations suggest a similar mechanism for the TCDD-dependent regulation of cytochrome P_1-450 gene expression in both rodent and human cells.

Based largely on studies carried out in murine skin (*14*), it has been postulated that TCDD-induced alterations in epithelial growth and differentiation are the consequence of the *Ah* receptor-dependent regulation of a second gene battery encoding growth regulatory proteins (*2,13*). Thus, the TCDD-dependent regulation of these genes would represent the primary response (or signal transduction event) resulting in epithelial growth dysfunction. Preliminary findings in this laboratory suggest that TCDD-dependent adverse growth patterns in cultured human keratinocytes require gene transcription. Studies in progress are focused on the identification and cloning of the putative dioxin-response growth control gene(s). Elucidation of the mechanisms of TCDD toxicity in the human epithelial cell culture models established in this and other laboratories should further understanding of the cellular and molecular determinants of human susceptibility to the HACs.

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